Ph.D. THESIS

Data on the ectomycorrhizal community of the „Őserdő" forest reserve (Bükk National Park, Hungary)

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I. Introduction and aims

In the majority of the climatic zones, the most important forest trees form ectomycorrhizae with different fungal species. These mycorrhizae are significant components of the ecosystems. Though the physiological base of the individual association is the reciprocal change of nutrients (Smith and Read 1997), the role that ectomycorrhizae play in the community is far more manifold: they influence the ecological relationships of species being in interaction with both the plant and the fungus (van der Putten et al. 2001), as well as they interconnect the members of the community into a common physiological entity (Selosse et al. 2006).

Although the ectomycorrhizal symbiosis was discovered in the late 19th century, the intense research on this association began only in the second half of the last century. Subsequent to the international surveys, studies of ectomycorrhizal communities started in Hungary in the 1990's. In addition to describing some specific ectomycorrhizal associations, general evaluation of mycorrhizae of different plant communities on the Great Hungarian Plain were also accomplished (Jakucs 2002, Kovács és Bagi 2001, Kovács és Szigetvári 2002).

The survey on the ectomycorrhizal community of the virgin beech forest of the "Őserdő" forest reserve (Bükk National Park) began in 2002 at the Department of Plant Anatomy (Eötvös Loránd University), as a part of a project (OTKA: Hungarian Research Fund). Previous sporocarp-based studies detected the macrofungi of the area (Siller 1986, 2004). Being an important group of woodlands in the temperate climatic zone, the ectomycorrhizal community of several beech forests were already examined in Europe, which provides the possibility of comparing the ectomycorrhizal composition of the first examined central European, continental beech stand with the results of other authors.

The basic aim of my Ph.D. work was to gather information on the ectomycorrhizal community of the study site by morpho-anatomical and molecular taxonomical methods. To reach this, I set up four different goals:

I. Following the identification of the most important types of ectomycorrhizae in the community, those were selected for further investigations, which were less-known from previous studies (genera *Genea*, *Humaria*, *Pachyphloeus*, *Tomentella*). These types were characterized in details by microscopic methods and their mycobionts were identified by DNA-sequence analysis. My specific questions were as follows:
   a) What species represent these genera in the ectomycorrhizal community?
   b) What are the phylogenetic relationships between the representatives of the genera?
   c) Which morphological-anatomical traits of the ectomycorrhizae distinguish the morphotypes from each other, from other specimens found in Hungary and from the previously described ectomycorrhizae of the genera?

II. In case of the less frequent ectomycorrhizal morphotypes, identified by molecular taxonomic methods (*Clavulina*, *Entoloma*, *Inocybe*, *Sebacina*, *Tricholoma*, *Tuber*), the aims were to briefly characterize the morphology and anatomy of the ectomycorrhizae, and to investigate the phylogenetic relationships of the mycobionts.

III. One of the *Lactarius* ectomycorrhizae of the area was regularly colonized secondarily by the hyphae of an alien ascomycetous fungus. My specific questions on this tripartite association were:
   a) What are the characteristic morphological and anatomical traits of the ectomycorrhiza, and what can we state about the identity of the mycobiont?
   b) What are the light and electronmicroscopical features of the alien hyphae colonizing the plant cortical cells of the ectomycorrhiza?

IV. I was to depict an overall view on the ectomycorrhizal community of the "Őserdő" forest reserve, based on the information gathered up to now. I compared the species composition and abundances of the species to the ectomycorrhizae of other deciduous forests in Hungary, and the communities of other European beech forests.

II. Materials and methods

Sampling the ectomycorrhizal community of the montane beech woodland of the "Őserdő" virgin forest (Bükk National Park) was carried out between 2002 and 2008**.** Soil cubes of 20 cm x 20 cm x 20 cm size were taken randomly from areas of different stand age. Soil samples were stored at 4°C for not more than two weeks.

Sample preparation and characterization of the mycorrhizae were accomplished by the method of Agerer (1991). Ectomycorrhizae of different morphology were separated into morphotypes under dissecting microscope, and they were fixed in FEA solution for further microscopy and in CTAB-solution for the molecular taxonomic analyses. Abundances of the morphotypes were estimated by a semi-quantitative method (Jakucs 2002), and given as the percentage proportion of the ectomycorrhizal tips to the total number of colonized root tips in the sample. On the basis of the percentage values, abundance categories were set up.

Microscopic investigations

Morphological and anatomical characterization of the ectomycorrhizae was principally approached by light microscopy (stereomicroscopy, Nomarski-DIC microscopy, phase-contrast microscopy). We investigated the whole fungal mantle removed from the root surface, as well as semi-thin (10 μm) longitudinal cryo-sections of the mycorrhizae stained with aniline-blue (with bright-field microscopy), and also 1-μm-thick unstained sections (using phase-contrast) embedded in Historesin. Histochemical reagents ($FeSO_4$-, guaiac-, sulpho-vanillin-, KOH-solution, lactic acid, Melzer-reagent) were applied for the characterization of some mycorrhizae. Additionally, the association between the *Lactarius*-ectomycorrhiza and the colonizing alien hyphae was characterized by transmission electron microscopy. For this purpose, samples were fixed in glutar-aldehyde and OsO_4-solutions, embedded in Durcupan resin, then stained with uranyl-acetate and lead-citrate.

Ectomycorrhizae of the genera *Humaria* and *Genea* were compared to each other by means of **morphometry**. Certain quantitative data of the mycorrhizae were measured, and statistically compared by ANOVA and Welch-test (d-test). The originally described (Jakucs et al. 1998) herbarium ectomycorrhiza specimen of *G. verrucosa* was also involved in this analysis.

Molecular analysis

DNA-extraction was carried out with the modified CTAB-method of Gardes and Bruns (1991). DNA was extracted from ectomycorrhizal root tips cleaned from any contaminating hyphae and spores. Different regions of the ribosomal DNA (rDNA) was amplified by **PCR** with sequence-specific and fungal specific primers; internal transcribed (ITS-)region was amplified for all ectomycorrhizae, 18S rDNA (SSU) for *Humaria*- and 28S rDNA (LSU) for *Pachyphloeus*-ectomycorrhizae. Subsequent to purification, the DNA-regions were **sequenced** with the primers used in the PCR. Homologous sequences were retrieved from molecular databases (GenBank, UNITE), and then aligned to the sequences of our samples applying the ClustalW software.

Phylogenies were inferred using Neighbor-joining (NJ) and Bayesian analyses in case of all ectomycorrhizae. This was completed by maximum parsimony (MP) when identifying the mycobiont of the *Lactarius* ectomycorrhiza, and by MP and maximum likelihood (ML) analyses for the genera *Humaria*, *Genea* and the *Pachyphloeus*-lineage. NJ and MP methods were conducted with Paup* 4.0 beta program (Swofford 2003), the ML analysis was inferred by PhyML (Guindon and Gascuel 2003), Bayesian trees were constructed by MrBayes 3.1.1 (Huelsenbeck és Ronquist 2001, Ronquist és Huelsenbeck 2003). The clades of the phylogenetic trees were statistically tested by bootstrap in case of NJ, MP and ML analyses.

In order to aid the identification of *Humaria*-, *Genea*- and *Lactarius*-ectomycorrhizae, the analysed regions of the rDNA were also acquired from **herbarial sporocarp samples**. These sequences were also involved in the phylogenetic reconstructions.

III. Results and discussion

At 10 sampling occasions, 30 soil samples were collected from the study site. Altogether 325 ectomycorrhizae were separated. 186 ectomycorrhizae were identified – at least – at the genus level: 66 of them by DNA-sequence analysis, and the rest based on their morphological-anatomical traits. These are the first results on a Hungarian ectomycorrhizal community acquired by both microscopical and molecular taxonomical approaches.

Ectomycorrhizae of the genera *Humaria* and *Genea*

Based on 12 ectomycorrhizal samples, identified by molecular methods as *Humaria*, we presented the first detailed description of the ectomycorrhiza of this genus. It was proved that

the former description of the *H. hemisphaerica* ectomycorrhiza was not formed by this species, which makes subsequent ecological studies applying this description, as a basis for anatomical identification, questionable.

Additionally, the presence of a *Genea* species was also proved in the ectomycorrhizal community, providing the possibility of comparing the mycorrhizal anatomy of the two genera. We detected no qualitative difference between the ectomycorrhizae of the epigeous *Humaria* and hypogeous *Genea* genera, yet the existence of morphometric differences was proved. By evaluating the quantitative characters, we found that the identification of the ectomycorrhiza originally described as *G. verrucosa* (Jakucs et l. 1998) is also ambiguous.

Ectomycorrhizae formed by species of the *Pachyphloeus-Amylascus* lineage

The mycobionts of three morphotypes have been identified as species related to the *Pachyphloeus-Amylascus* lineage. We have characterized and documented the morphology and anatomy of these ectomycorrhizae in details, and outlined the differences between the three morphotypes.

According to the results of the rDNA-sequence analyses, the three morphotypes are formed by three different species of the lineage. One of them can be identified as *P. melanoxanthus* and another can be regarded as an unidentified *Pachyphloeus* species. In case of the third morphotype, we can only tell that it is closely related to the *Pachyphloeus-Amylascus* lineage, without identifying even the genus. However, the ectomycorrhizae of neither species was observed previously.

Tomentelloid ectomycorrhizae

Six different tomentelloid morphotypes (ten ectomycorrhizae, altogether) were found as members of the community. Our molecular results proved that these were formed by six species of the genus *Tomentella*. The anatomical and molecular comparison to the tomentelloid ectomycorrhizae of other Hungarian regions revealed that five morphotypes were found only in the "Őserdő".

In case of two morphotypes, the mycobiont can be identified at the species level (as *T. stuposa* and *T. ramosissima*), and another as "probably" *T. sublilacina*. However, in case of the other three types only the phylogenetic position within the genus can be stated.

On the grounds of anatomical comparisons, we presented the specific characters distinguishing the morphotypes from each other, and from the previously described tomentelloid ectomycorrhizae.

Further identified components of the ectomycorrhizal community

In addition to the above mentioned genera, further 16 members of the ectomycorrhizal community were identified on the bases of DNA-sequences. The phylogenetic analyses proved that these mycorrhizae were formed by 13 different species.

Two morphotypes are the mycorrhizae of the genus *Clavulina*. One of these can be identified as *C. cristata*. The mycobiont of another type is related to *Entoloma sinuatum*, yet it cannot be assigned accurately to any species. Four morphotypes were formed by *Inocybe* spp., three of which can be identified to the species level (*I. asterospora*, *I. fuscidula* and *I. petiginosa*). The ectomycorrhiza of *I. asterospora* and *I. fuscidula* were not indicated previously.

The mycobionts of three ectomycorrhizal morphotypes belong to the genus *Sebacina*, a basal group of Basidiomycetes with phragmobasidia. The three morphotypes have distinct characteristic anatomical features. Since there are insufficient identified sequences of this genus in the molecular databases, we cannot identify the ectomycorrhizae at the species level.

We have also found the ectomycorrhizae of *Tricholoma lascivum* and one related to the *T. bufonium – T. sulphureum* group. Ectomycorrhiza of the first species was never indicated in the literature before.

We have also identified the mycorrhiza of *Tuber puberulum* from the study site, though this is not an original result, since Kovács and Jakucs (2006) have already mentioned the presence of this species in the ectomycorrhizal community.

In case of all ectomycorrhizae, the morphological and anatomical similarity and differences between the morphotypes and other ectomycorrhizae of the genera were evaluated, and we tended to find relations between them and the phylogenies inferred.

Characterization of the *Lactarius*-ectomycorrhiza colonized by alien hyphae

In the "Őserdő" we regularly found the *Lactarius*-ectomycorrhiza of characteristic morphology and anatomy, colonized always by alien hyphae of an ascomycete. The abundance of this morphotype was usually rather high; often this was the dominant ectomycorrhiza in the soil samples. We have phylogenetically analysed nine ectomycorrhiza-

sequences together with ones obtained from sporocarps collected in the study site, and those from molecular databases. As a result of these analyses, the mycobiont can be identified as *L. subdulcis* (subg. *Russularia*). The morphology and anatomy of the ectomycorrhiza was described in details, and documented. Most of the observed features are similar to those described by Brand (1991) on the ectomycorrhiza of the species, yet we found differing traits that characterized all our samples. The anatomy of the mycorrhiza was compared to that formed by other species of the subgenus.

In all the collected ectomycorrhizae, the cortical plant cells below the fungal mantle were intracellularly colonized by alien, ascomycetous hyphae. We examined and documented this tripartite symbiosis by light and electron microscopy. Compared to the previously described endogenous hyphae colonizing *Lactarius* ectomycorrhizae, the observed colonization showed the highest similarity to the ascomycetous hyphae found by Pargney and Prévost (1996) in the ectomycorrhizae of *L. subdulcis*.

Comparative evaluation of the ectomycorrhizal community of the study site

The community of "Őserdő" – similarly to the general structure of ectomycorrhizal communities (Horton and Bruns 2001) – composes of a few abundant and several less frequent morphotypes. Though the structure of the community has not been completely resolved yet, some general conclusions can be drawn from the results gathered up to now.

Beside the very frequent light coloured ectomycorrhizal morphotypes (*Lactarius*, *Russula* and the boletoid species) the dark-mantled ones (tomentelloids, *Cenococcum geophilum*, *Genea*, *Humaria*) are subdominant. This result is just the opposite that was observed in the Hungarian deciduous forests of the Great Plain, where the dominance of tomentelloid species was characteristic (Jakucs 2002).

Comparing the ectomycorrhizal community of the "Őserdő" to those of other European beech forests, we found that the characteristic genera are the same, but their abundance values may considerably differ among the stands.

IV. Publications in the topic of the dissertation

Articles:

Erős-Honti[1] Zs., Jakucs E. (2009): Characterization of beech ectomycorrhizae formed by species of the *Pachyphloeus–Amylascus* lineage. *Mycorrhiza* (on-line available) DOI 10.1007/s00572-009-0236-3

Jakucs E., Ganyec Sz., Erős-Honti Zs. (2008): "*Fagirhiza asteromustrata*"+ *Fagus sylvatica* L. *Descriptions of Ectomycorrhizae* 9/10:31-35.

Jakucs E., Erős-Honti Zs. (2008): Morphological-anatomical characterization and identification of Tomentella ectomycorrhizas. *Mycorrhiza* 18(6-7):277-285.

Erős-Honti Zs., Kovács G.M., Szedlay Gy., Jakucs E. (2008): Morphological and molecular characterization of *Humaria* and *Genea* ectomycorrhizae from Hungarian deciduous forests. *Mycorrhiza* 18: 133-143.

Jakucs E., Kovács G.M., Szedlay Gy., Erős-Honti Zs. (2005): Morphological and molecular diversity and abundance of tomentelloid ectomycorrhizae in broad-leaved forests of the Hungarian Plain. *Mycorrhiza* 15: 459-70.

Jakucs E., Kovács G.M., Agerer R., Romsics Cs., Erős Zs. (2005): Morphological-anatomical characterization and molecular identification of *Tomentella stuposa* ectomycorrhizae and related anatomotypes. *Mycorrhiza* 15: 247-258.

Posters:

Jakucs E., Kovács G.M., Erős-Honti Zs. (2008): Study of species composition of the ectomycorrhizal community of beech forests in Hungary. IV. Magyar Mikológiai Konferencia, Debrecen. (Abstract: *Acta Microbiologica et Immunologica Hungarica* 55: 201 p.)

Erős-Honti Zs., Jakucs E., Kovács G.M. (2006): Ectomycorrhizae of *Genea* and related genera from Hungarian broad-leaved forests. 5th International Conference on Mycorrhiza, Granada. p. 122.

Jakucs E., Kovács G.M., Erős-Honti Zs. (2006): Morphological characteristics and molecular diversity of *Tomentella* ectomycorrhizae in deciduous forests. 5th International Conference on Mycorrhiza, Granada. p. 128.

Jakucs E., Erős-Honti Zs., Szedlay Gy. (2005): *Genea*-ektomikorrhizák hazai erdőtársulásokból. [*Genea* ectomycorrhizae from Hungarian woodlands] III. Magyar Mikológiai Konferencia, Mátraháza. (Abstract: *Acta Microbiologica et Immunologica Hungarica* 52: 230 p.)

Erős Zs., Kovács M.G., Jakucs E., Keresztes Á. (2003): Hármas szimbiózis a bükk (*Fagus sylvatica*) egy *Lactarius* fajjal képzett ektomikorrhizája és egy intracelluláris kolonizáló tömlősgomba között. [Tripartite symbiosis between the ectomycorrhiza formed by beech (Fagus sylvatica) with a *Lactarius* species and an ascomycete colonizing the cortical plant cells] VI. Magyar Ökológus Kongresszus, Gödöllő – p. 82.

Jakucs E., Kovács M.G., Erős Zs., Király I. (2003): Anatomical and molecular characterization of the ectomycorrhizae of *Tomentella stuposa* (Thelephoraceae, Basidiomycetes). 14th International Congress of the Hungarian Society for Microbiology, Balatonfüred.

[1] Since 2005, my publications have been issued under the name Zsolt Erős-Honti, instead of my birth-given name (Zsolt Erős).

Lectures:

Erős-Honti Zs., Kovács G. M., Szedlay Gy., Jakucs E. (2008): A *Humaria* és *Genea* nemzetségek ektomikorrhizái magyarországi lombhullató erdőkben**. [**Ectomycorrhizae of the genera *Humaria* and *Genea* from Hungarian deciduous forests.**]** (Abstract: Acta Microbiologica et Immunologica Hungarica 55: 187 p.)

Erős-Honti Zs., Jakucs E. (2006): Adatok a bükki Őserdő ektomikorrhiza-közösségéről. [Details of the ectomycorrhizal community of the „Őserdő" forest reserve (Bükk National Park, Hungary)] 7. Magyar Ökológus Kongresszus, Budapest. p. 58.

Erős-Honti Zs., Jakucs E., Szedlay Gy., Kovács M.G. (2005): A bükki Őserdő ektomikorrhiza-közösségének vizsgálata. [Survey ont he ectomycorrhizal community of the „Őserdő" forest reserve (Bükk mountains).] III. Magyar Mikológiai Konferencia, mátraháza. (Abstract: *Acta Microbiologica et Immunologica Hungarica* 52: 201 p.)

V. Other publications

Articles:

Vasas G., Erős-Honti Zs. (2009): Két Magyarország területéről leírt *Agaricus* faj összehasonlító morfológiai és molekuláris biológiai vizsgálata. [Morphological and molecular taxonomical comparison of two *Agaricus* species described from Hungary.] *Mikol Közl*. (közlésre elfogadva)

Erős-Honti Zs., Jakucs E., Kovács M.G. (2008): "*Helianthemirhiza ochraceo-brunnescens*" + *Helianthemum canum* (L.) Baumg. *Descriptions of Ectomycorrhizae*. 11/12:71-75.

Erős Zs. (2003): Ektomikorrhiza-kapcsolatok a törpecserjéket magába foglaló szuharfélék (*Cistaceae*) családjában. [Ectomycorrhizae of the family Cistaceae.] *Mikol. Közl.* 42: 35-44.

Posters:

Kutszegi G., Dima B., Erős-Honti Zs., Jakucs E. (2008): Distribution and characterization of Bankeraceae (Basidiomycota) species in Hungary. IV. Magyar Mikológiai Konferencia, Debrecen. (Abstract: *Acta Microbiologica et Immunologica Hungarica* 55: 213 p.)

Erős-Honti Zs., Jakucs E. (2004): A Thelephorales rend molekuláris taxonómiai vizsgálata. [Molecular taxonomic study on the order Thelephorales.] A Magyar Mikrobiológiai Társaság 2004. Évi Nagygyűlése, Keszthely

Erős Zs., Jakucs E., Kovács M.G. (2004): Ektomikorrhizák leírása a szürke napvirágról (*Helianthemum canum* (L.) Baumg.) [Ectomycorrhizae of the hoary rockrose (*Helianthemum canum* (L.) Baumg.)] Aktuális Flóra- és Vegetációkutatás a Kárpát-medencében VI. Előadások és poszterek. Keszthely – p.44.

Erős Zs., Jakucs E., Kovács M.G. (2003): Ectomycorrhizal status of the hoary rockrose (*Helianthemum canum* (L.) Baumg.) 14th International Congress of the Hungarian Society for Microbiology, Balatonfüred.

Lecture:

Jakucs E., Kovács M. G., Erős-Honti Zs. (2006): A mikorrhizák ökológiai jelentősége. [Ecological significance of ectomycorrhizae] 7. Magyar Ökológus Kongresszus, Budapest. p. 10.